

FEDEROFF DECLARATION

Exhibit C

Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain

Michael G. Kaplitt^{1,2}, Paola Leone¹, Richard J. Samulski³, Xiao Xiao⁴, Donald W. Pfaff², Karen L. O'Malley⁵ & Matthew J. During¹

Adeno-associated viral (AAV) vectors are non-pathogenic, integrating DNA vectors in which all viral genes are removed and helper virus is completely eliminated. To evaluate this system in the post-mitotic cells of the brain, we found that an AAV vector containing the *lacZ* gene (AAVlac) resulted in expression of β -galactosidase up to three months post-injection *in vivo*. A second vector expressing human tyrosine hydroxylase (AAVth) was injected into the denervated striatum of unilateral 6-hydroxydopamine-lesioned rats. Tyrosine hydroxylase (TH) immunoreactivity was detectable in striatal neurons and glia for up to four months and we also found significant behavioural recovery in lesioned rats treated with AAVth versus AAVlac controls. Safe and stable TH gene transfer into the denervated striatum may have potential for the genetic therapy of Parkinson's disease.

¹Molecular Pharmacology and Neurogenetics Laboratory, Department of Surgery, Yale University School of Medicine, New Haven, Connecticut 06520-8039, USA

²Laboratory of Neurobiology and Behavior, The Rockefeller University, New York, New York 10021, USA

³Gene Therapy Center, University of North Carolina, Chapel Hill, North Carolina 27599, USA

⁴Mertin Pharmaceuticals, New York, New York 10014, USA

⁵Department of Anatomy and Neurobiology, Washington University School of Medicine, St Louis, Missouri 63110, USA

Correspondence should be addressed to M.J.D.

The ability to manipulate the expression of genes within the mammalian brain provides unique opportunities to study and potentially treat neurologic disorders. Vectors based upon DNA viruses are most appropriate for transduction of post-mitotic cells within the brain, since retroviral vectors only function within dividing cells¹. Recombinant herpes simplex virus type 1 (HSV) was originally used for gene transfer into neurons in tissue culture and *in vivo*^{2,3}, as HSV is a naturally neurotropic virus. Typically, these vectors carry deletions in viral genes which either limit the toxicity of replicating viruses or render the vectors replication defective, thereby decreasing pathogenicity. However, they retain numerous functional viral genes, which may be cytotoxic to the recipient cells and which can potentially reactivate latent viruses which exist within most adults^{4,5}. More recently, recombinant adenoviral vectors have been used as neuronal gene transfer vehicles but these also retain viral genes which can produce viral proteins within recipient cells⁶⁻⁹.

In order to eliminate viral gene expression, defective viral vectors have been developed. For example, defective HSV vectors contain an HSV origin of DNA replication and a cleavage/packaging signal, which are recognition sequences, but no viral genes. In the presence of a helper virus, the plasmid is replicated and packaged into an HSV particle, creating a defective viral vector^{10,11}. Previously, we and others have demonstrated that defective HSV vectors can transfer foreign genes into the adult central nervous system (CNS) *in vivo*¹²⁻¹⁴. Although HSV mutants have been used as helper viruses¹²⁻¹⁵, however, issues of continued helper virus gene expression, pathogenicity and reversion to wild-type still remain.

The adeno-associated viral (AAV) vector system is a new approach to gene transfer in the CNS. Wild-type AAV is a nonpathogenic parvovirus which is incapable of autonomous replication and spread. A productive infection requires co-infection by a non-AAV helper virus (either adenovirus or HSV) which provides proteins necessary for AAV replication^{16,17}. In the AAV vector system, 96% of the parental genome has been deleted such that only the terminal repeats remain, containing recognition signals for DNA replication and packaging. AAV structural proteins are provided *in trans* by co-transfection of the AAV vector with a helper plasmid containing the missing AAV genes but lacking replication/packaging signals^{18,19}. Following infection with adenovirus, two populations of particles are obtained: progeny helper adenovirus and AAV vectors encoding foreign genes; the helper plasmid lacking AAV terminal repeats is not packaged. Since the AAV coat proteins are structurally distinct from the helper adenovirus, contaminating adenovirus particles can be completely removed. The AAV vector is thus unique among current DNA viral vectors, as it contains only the gene of interest with no viral genes and is completely free of contaminating helper virus. An important basis for pathogenicity is thereby eliminated, rendering this system particularly suited to human gene therapy.

One CNS disorder which may be amenable to gene transfer techniques is Parkinson's disease (PD). PD is characterized by loss of the nigrostriatal pathway and is responsive to treatments which facilitate dopaminergic transmission in the caudate-putamen^{20,21}. In rodent models of PD, mesencephalic fetal cells or genetically modified cells expressing tyrosine hydroxylase (TH) synthesize

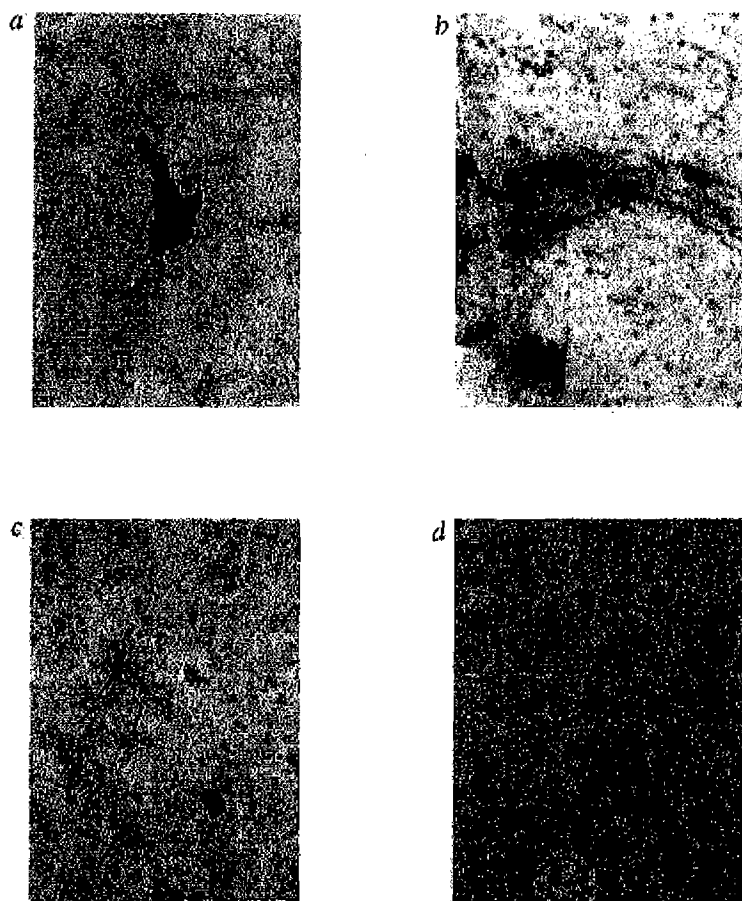


Fig. 1 Transfer of the *lacZ* gene into rat brain via an AAV vector. **a** and **b**, Staining for β -galactosidase histochemistry three days after injection of AAVlac. Positive cells are seen within the hippocampus (**a**) and lateral cortex (**b**), with processes suggestive of neuronal staining particularly evident in the lateral cortex. **c**, β -galactosidase expression in the caudate nucleus three months after injection of AAVlac, demonstrating the continued presence of enzymatically active foreign gene product. **d**, Demonstration of the presence of *lacZ* DNA in caudate nucleus two months following injection by *in situ* PCR. Staining was not observed in the opposite caudate nor in sections from animals injected with adenovirus dl309. All sections were 30 μ m thick and were counterstained with hematoxylin and eosin. Magnification: **a**, 100 \times ; **b-d**, 400 \times .

dihydroxyphenylalanine (L-Dopa), which induces behavioural recovery²²⁻²⁵. Moreover, direct gene transfer into the denervated striatum of lesioned animals with defective HSV vectors encoding TH can also be successful (M.J.D., *et al.*, manuscript submitted).

We now report the successful application of the AAV vector system in transducing long term *lacZ* gene expression in various brain regions. Furthermore, long term TH expression was demonstrated in the denervated striatum, and significant behavioural recovery was observed in lesioned animals with no evidence of pathogenicity.

AAV vector for gene transfer into brain

An AAV plasmid encoding the *lacZ* gene was created by subcloning the human cytomegalovirus (CMV) immediate-early promoter, *lacZ* and an SV40 polyadenylation signal between the terminal repeats of the AAV genome in plasmid psub201 (ref. 19). These termini contain the recognition signals necessary for replication and packaging into an AAV vector. Cells were co-transfected with pAAVlac and pAAV/Ad, and then infected with adenovirus type 5 (strain dl309)²⁶ to provide remaining functions necessary for AAV replication (see Methodology).

AAV vectors were titrated by infection of cultured 293 cells followed by histochemical staining for β -galactosidase expression and counting of the resulting blue cells. There was no difference in the number of cells observed at one

and five days following infection, demonstrating an absence of vector replication and spread. When the process was repeated using a *lacZ* plasmid without the AAV recognition signals, no positive cells were observed following infection with the resulting stock. The complete elimination of adenovirus was confirmed by the inability to detect any viral plaques in cultured cells one week following infection with this viral stock. This indicates that the *lacZ* gene was packaged into an AAV virus which was incapable of autonomous replication while residual adenovirus was completely eliminated.

AAV-mediated gene expression in adult rat brain

AAVlac was stereotactically microinjected into various regions of the adult rat brain, including caudate nucleus, amygdala, striatum and hippocampus. Initially, animals were killed between one and three days following injection and sections were processed for β -galactosidase expression via X-gal histochemistry. Positive cells were found within each region (Fig. 1a and b). The efficiency of gene transfer into the brain appeared to be at least 10%, which is equivalent to previous observations with HSV or adenovirus vectors^{2,3,6-8,12-14}, and with an earlier observation of short-term expression from an AAV vector in the brain (Xiao, X. & Samulski, R.J., manuscript submitted).

In order to analyse the long-term stability of AAV gene transfer and expression within the mammalian brain, animals were injected in the caudate nucleus with AAVlac and killed 2-3 months following surgery. Sections were

processed for X-gal histochemistry, using a technique which completely eliminates background staining^{12,13}, in order to identify cells containing functional β -galactosidase. Positive cells were identified within injected regions of the caudate nucleus up to 3 months following vector injection (Fig. 1c). Additional animals were then killed and tissue sections from these animals were examined using the polymerase chain reaction (PCR), which was

modified to permit amplification and visualization of viral vector DNA *in situ*²⁷⁻²⁹. Numerous cells within the brain were detected which retained the bacterial *lacZ* gene after two months (Fig. 1d). There was no staining on the contralateral side, in sections processed without *Taq* polymerase or in sections from brains injected with adenovirus alone (data not shown). At no time were behavioural or physiological abnormalities detected within

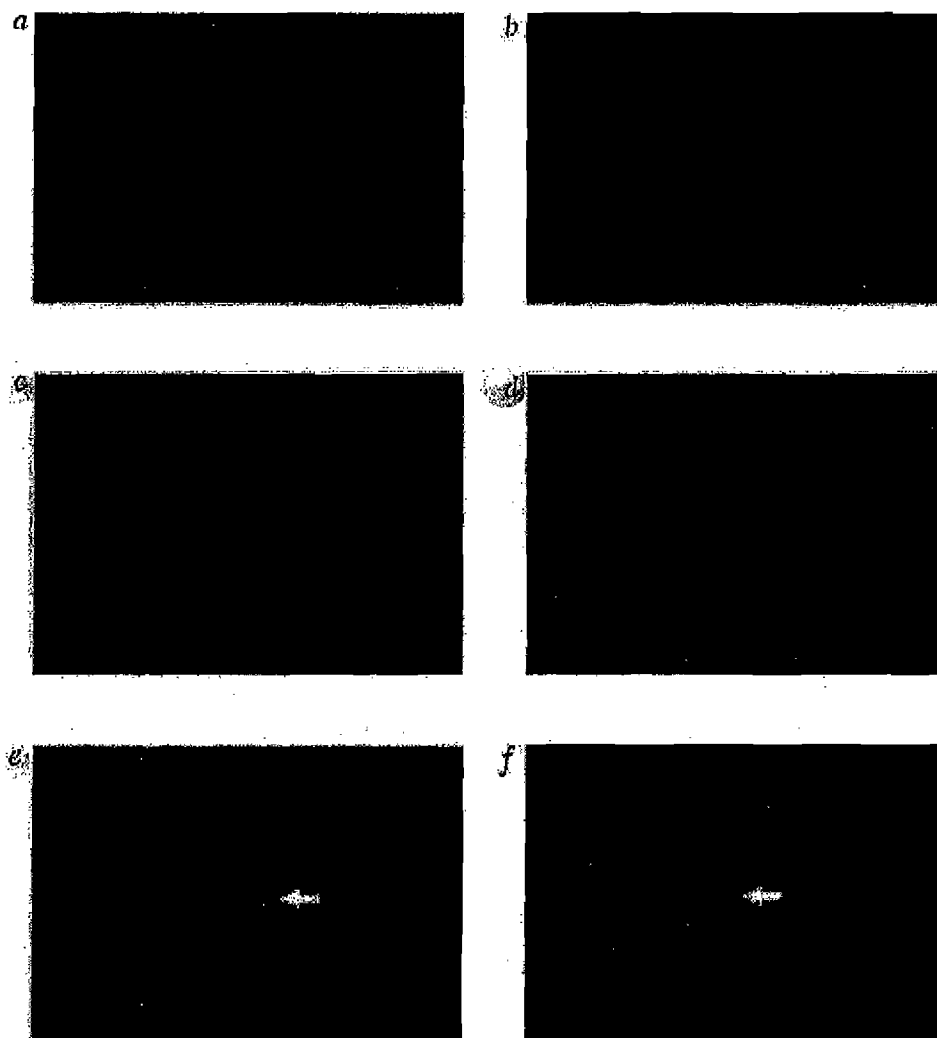


Fig. 2 Immunohistochemical detection of human TH expression within the caudate nucleus of 6-OHDA lesioned rats following injection of AAVth. *a*, Absence of immunostaining in caudate following injection of AAVlac. No staining was ever observed in AAVlac animals, and staining was also always absent from the uninjected caudate from AAVth animals. *b* and *c*, TH expression in cells of the caudate nucleus four months after injection of AAVth. These sections were 30 μ m thick, which prevented morphological identification of positive cells. Approximately 30 cells are seen at the site of injection (*b*) and cells are also seen 2 mm away from the injection site (*c*), although fewer cells are present at 2 mm. This observation was repeated twice at four months following injection, while comparable results were obtained from three animals at two months and two animals at one month following injection. *d*, TH expression in caudate one week following AAVth injection. This section was 7 μ m thick, revealing the neuronal appearance of the majority of positive cells. 50 positive cells can be seen in this section, which is representative of approximately 50 consecutively positive sections obtained from each short-term animal. Fewer cells were observed as far as 280 sections (2 mm) away from the injection site. This result was repeated twice at one week following injection, and comparable results were obtained from nine animals at 48 h and nine animals at 24 h post-injection. *e* and *f*, Double-label immunocytochemistry demonstrating neuronal TH expression. *e*, TH expression in a caudate cell (arrow) was revealed using a FITC-labelled secondary antibody. *f*, Neuronal identification of the TH-expressing cell (arrow) was obtained by sequentially staining the same section with an anti-neurofilament antibody and visualization with a Texas red-conjugated secondary antibody. Magnification: *a-d*, 400 \times ; *e* and *f*, 630 \times .

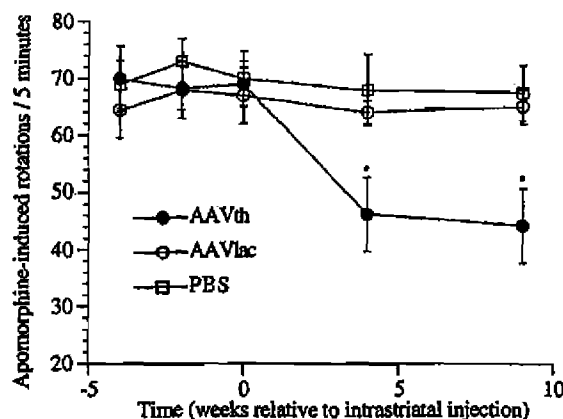


Fig. 3 Effect of AAVth injection upon rotational behavior of 6-OHDA lesioned rats. Rotational behaviour was induced in lesioned rats by administration of apomorphine. Rats were tested over a period of at least six weeks prior to injection of AAV vectors in order to obtain consistent baseline rotational rates. Animals injected with AAVlac ($n=3$) as well as PBS-injected animals ($n=4$) exhibited no change in rotational rate throughout the two month test period following surgery. Subjects treated with AAVth ($n=4$) demonstrated a significant decrease in rotational rate compared with baseline, PBS or AAVlac treated animals ($p<0.02$) reaching 38% by two months following vector injection. Asterisks indicate time points which yielded significant improvement compared with PBS and AAVlac treated controls ($p<0.05$) as determined by post-hoc analysis and error bars are standard errors.

the animal subjects, and the brain sections showed no evidence of pathology resulting from the AAV gene transfer.

Human TH expression in 6-OHDA lesioned rats

In order to generate vector AAVth, which may have therapeutic utility in human Parkinson's disease (PD) patients, the *lacZ* gene was replaced with a human TH cDNA (form II)³⁰. AAVth was packaged and helper virus was eliminated as before (see Methodology). Following injection of AAVth into the denervated striatum of rats with substantia nigra lesions (see below), animals were analysed for TH expression from 24 hours up to four months (Fig. 2). Expression of TH from the AAV vector was detected using immunocytochemistry with a mouse monoclonal anti-TH antibody. Although this antibody does not distinguish between the rat and human protein, TH is not expressed within either the intrinsic neurons or glia of the rat striatum³¹. Furthermore, endogenous TH immunoreactivity (TH-IR) is limited to the dopaminergic afferent fibres within the striatum of unlesioned animals, and is therefore completely absent in the fully denervated striatum. In both control, uninjected rats and AAVlac-injected rats there was no striatal TH immunoreactivity (TH-IR) on the denervated side (Fig. 2a). In contrast, in the denervated striata injected with AAVth, numerous TH-IR cells were clustered around the injection site (Fig. 2b and d) and extending to two mm away from the injection (Fig. 2c). No TH-IR cells were identified on the contralateral, uninjected side of these sections. The majority of cells within the striatum appeared to be neurons morphologically, and double-labelling with both the anti-

TH monoclonal antibody and an anti-neurofilament antibody confirmed that a substantial number of striatal neurons expressed immunoreactive TH *de novo* (Fig. 2e and f). Additional sections were double labelled with an antibody to glial fibrillary acidic protein (GFAP), which also demonstrated TH positive glial cells (data not shown). Thus, following gene transfer into the rat striatum via an AAV vector, the majority of TH-IR cells in the striatum were neurons while a small percentage of TH-IR cells were glial cells.

Striatal TH expression was also examined at times ranging from three days to four months following injection of AAVth. Expression persisted throughout this four month period (Fig. 2b and c), although the level of expression, as manifest by the number of positive cells, did appear to diminish. Furthermore, gene transfer appeared to be safe in the experimental subjects, as there were no signs of cytopathic effects in any animal (AAVth or AAVlac) at any time. The only changes observed in the short term AAVth animals (examined less than one week following injection) was a slight needle injury at the injection site, which was similar in PBS-injected and AAVlac-injected animals. In the long-term animals (greater than two months), the residual needle track was not consistently visible and there was no evidence of any neuronal injury or reactive gliosis. A total of 12 rats (AAVlac and AAVth) were studied for two months or longer and 37 rats (AAVlac and AAVth) were studied for variable times from 24 hours to one month, and there were no behavioural or gross pathological signs of brain damage and no deaths prior to experimental sacrifice in any subject.

AAVth promotes behavioural recovery

Unilateral 6-hydroxydopamine (6-OHDA) lesions of the substantia nigra have been used to generate an established rodent model of PD. In this model, the asymmetry caused by differing postsynaptic receptor sensitivities between the denervated and intact striatum results in rotational behaviour following systemic administration of dopaminergic agents, such as the direct acting agonist, apomorphine³². The rate of asymmetrical rotation is directly related to the severity of the striatal dopamine deficit and this model has predictive ability in defining treatments which may have therapeutic efficacy in PD^{33,34}. Lesioned rats were tested for apomorphine-induced rotation every two weeks on a minimum of three occasions, and animals that satisfied behavioural criteria of >90% lesion efficacy were identified³². AAVth or AAVlac virus, or PBS, was delivered by stereotactic injection into the denervated striatum. Animals were tested for apomorphine-induced asymmetrical rotation at one and two months. The rotational behaviour of the AAVlac and PBS injected animals were unaltered from baseline. In contrast, AAVth injected animals ($n=4$) demonstrated significant behavioural recovery (Fig. 3), compared to both the AAVlac-injected ($n=3$) or PBS-injected ($n=4$) groups ($p<0.02$; repeated measures ANOVA). The average behavioural recovery caused by AAVth was $33 \pm 9\%$ at one month and was maintained at $36 \pm 9\%$ two months after injection, and post-hoc analysis indicated that both time points were statistically significant compared with baseline and controls ($p<0.05$).

Discussion

We report here the first demonstration that AAV vectors

can safely and stably transfer and express a potentially therapeutic gene in the adult rat brain. Furthermore, stability of viral DNA and *lacZ* expression within the brain was observed for at least three months. Expression of human tyrosine hydroxylase (hTH) was also demonstrated in rat brains which had previously received unilateral 6-OHDA lesions in the substantia nigra. The lesions permitted unambiguous identification of cells expressing hTH within the rat caudate nucleus from three days to four months following vector injection. The majority of positive cells were morphologically neurons, which was confirmed by double-label immunocytochemistry, although hTH expression was also demonstrated within glial cells. AAV vectors were injected into a total of 12 long-term (at least two months) and 37 short-term subjects, and absence of objective indicators of pathogenicity or toxicity suggests that this system is safe as well as effective.

Using the 6-OHDA lesioned rodent model of PD, our study demonstrates that significant behavioural recovery can be achieved following AAVth injection. Other systems which have previously been used for this purpose, including implantation of heterologous cell types have varied widely, ranging from 30%–74% (refs 22, 24). While it is believed that L-Dopa is converted to dopamine *in situ*, the source of decarboxylase activity has yet to be identified. Generally, the remaining nigra-striatal afferents are the best candidates for this activity. The extent of the lesions in the animals used in the current study was such that few, if any, nigra-striatal terminals remained. Therefore, a lack of substantial endogenous decarboxylase activity may have limited the effect of AAVth. This suggests that in the future, constructs encoding both dopamine biosynthetic enzymes (TH and aromatic acid decarboxylase) may result in far greater improvements in behavioural recovery. This is supported by the fact that the best behavioural recovery reported so far (74%) has been observed with transplanted muscle cells containing a transfected TH gene, and muscle cells have endogenous decarboxylase activity²⁴.

The titre of the AAVth stock used for these *in vivo* studies was 5×10^6 infectious units/ml. Therefore a single injection of 2 μ l would result in 10,000 positive cells if the efficiency of infection was 100% and each particle infected a different cell. In the AAVth-injected animals, the total number of striatal cells containing TH-IR consistently exceeded 1,000 for each of the 2 μ l injections suggesting a minimum of 10% *in vivo* efficiency, significantly greater than our previous observations using defective HSV-1 vectors^{12,13}. Since previous infection of AAV does not prevent subsequent infection or multiple particles infecting the same cell³⁵, the actual efficiency may be higher. Alternatively, since the cellular receptor for AAV is unknown, the efficiency of infecting striatal cell types may be considerably less than determined with 293T cells.

Expression persisted for at least four months, however there was a decrease in the number of positive cells. While it is possible that vector genomes were lost from some cells or that some positive cells died, loss of foreign gene expression may more likely be due to the use of the CMV promoter. Earlier studies have shown a loss of transgene expression with time due to inactivation of genetic elements associated with some viral immediate early promoters^{13,36}. Therefore, endogenous cellular promoters may provide more uniform long-term expression. Although it appears that a greater percentage of TH-positive cells was observed in the current study when

compared with previous CNS gene transfer studies, so far there has been no systematic analysis of long-term expression in either the HSV, adenovirus or AAV systems and this clearly requires further examination. The fate of vector genomes within modified brain cells *in vivo* is at present unclear. Wild-type AAV can integrate within the chromosomal DNA of the host cell, a property unique among DNA viruses^{16,17}. In human cells, integration appears to be targeted to a specific site on chromosome 19 (19q13.3), although no specific site has yet been identified in rodent cells³⁷. The AAV vector can also integrate into human cells in culture, indicating that expression of AAV gene products is not required for integration³⁸. Furthermore, recent evidence has indicated that the AAV vector also can exhibit targeted integration into chromosome 19 in cultured haematopoietic stem cells³⁹. However, episomal copies of AAV vector genomes have also been demonstrated in some cultured human cells³⁸. No information exists so far on the fate of AAV vector genomes in rodent cells or in any species following *in vivo* gene transduction. Additional studies will be valuable to determine the fate of AAV vector DNA within host brain cells *in vitro* and *in vivo*, and the role, if any, of integration in maintaining long-term expression.

Our data suggest that AAV vectors may be a useful approach to alter in a safe and stable manner *in vivo* gene expression in neurons. Furthermore, AAV vectors expressing TH appear to have potential as a direct *in vivo* somatic cell gene therapy approach to Parkinson's disease. Further studies examining the efficacy of TH and other potentially therapeutic genes in rodent and primate disease models are in progress. Previous studies demonstrating gene transfer into the rodent CNS have used only marker genes or potentially toxic viral vectors, however, our current results indicate that safe and effective genetic therapy of CNS disease may be possible.

Methodology

Plasmids. Plasmid pSub201 (ref. 19) was digested with *Xba*I to remove nearly the entire AAV genome, leaving only the terminal repeats. A CMV promoter-*lacZ* gene-SV40 polyA signal cassette was isolated from plasmid pHCL¹³ by digestion with *Spe*I and *Xba*I, and this was inserted into *Xba*I-digested pSub201 to create pAAVlac. A second plasmid was created (pAAV-CMV-polyA) by digestion of pAAVlac with *Hind*III and *Xba*I to remove the *lacZ* gene and polyA signal, followed by insertion of a *Hind*III-*Xba*I fragment from pREP4 (Invitrogen), containing a polylinker and SV40 polyA signal. This plasmid was then digested with *Hind*III and *Bam*HI, followed by insertion of a human TH cDNA²⁰ in order to create pAAVth.

Creation of defective viral vectors. In order to create AAV vectors, plasmids (pAAVlac or pAAVth) were transfected via the calcium phosphate method⁴⁰ into 293T cells (obtained from D. Baltimore, Rockefeller University), a variant of 293 cells⁴¹ which constitutively express both the adenovirus E1a protein and the SV40 T antigen. The vector plasmids were co-transfected along with the helper plasmid pAAV/Ad¹⁹, which encodes AAV structural proteins. The next day, cells were infected with adenovirus strain dl309 (ref. 26) (obtained from T. Shenk, Princeton). Following full cytopathic effect, virus was harvested by multiple freeze/thaw cycles. Viral stocks were then heated to 56°C for 30 min in order to inactivate residual adenovirus¹⁹. Vector titres were obtained by histochemical assay for X-gal¹³ or immunocytochemical identification of hTH expression in 293T cells infected with serial dilutions of the vector stock, using a monoclonal anti-hTH antibody (Boehringer-Mannheim) and the ABC elite detection system (Vector labs).

Animals. Male Sprague-Dawley rats were used in all studies. Animals were treated according to the NIH Guidelines for Animal Care and

Use. A total of 49 animals were studied. 19 animals received AAVlac and 30 animals received AAVth. Seven of the AAVlac animals were studied for two months or longer (including three 6-OHDA lesioned rats used in the behavioural analysis). Five AAVth rats were studied at two months or longer (four with 6-OHDA lesions used for behavioural analysis). The remaining animals were studied at time points including 24 h ($n=3$ for AAVlac and $n=9$ for AAVth), 48 h ($n=2$ for AAVlac and $n=9$ for AAVth), 1 week ($n=3$ for both AAVlac and AAVth) and 1 month ($n=4$ for both AAVlac and AAVth).

AAVlac injection and X-Gal histochemistry. Animals were anaesthetized with a mixture of enflurane and NO_2 . Stereotaxic microinjection of AAVlac was used for all brain region injections, and coordinates were determined according to the atlas of Paxinos and Watson¹². Following sacrifice, brain tissue was fixed and stained with the histochemical substrate X-Gal, as described previously^{12,13}. 5mM EGTA was included in the fixative in order to completely eliminate staining due to endogenous cellular enzymes^{12,13}.

In situ PCR. Tissue was fixed in 4% paraformaldehyde in PBS (pH 7.3). Brain sections were pre-treated in detergent buffer (0.01% sodium deoxycholate/0.02% NP-40 in PBS) for 1 h. Following PBS wash, sections were dehydrated in alcohol and 200 μl of PCR reaction buffer was added to each slide (PCR reaction buffer: 1 \times PCR buffer/1 μM each primer/1 M MgCl_2 /10 μl digoxigenin-dUTP. Primers specific for the *lacZ* gene have been described¹³). Slides were coverslipped and coverslips were anchored on one side with nail polish. Slides were placed on aluminum foil on the block of a thermal cycler, and the temperature was raised to 82 $^\circ\text{C}$. Coverslips were raised, 2 μl of enzyme mix (1 \times PCR buffer/2 U ml^{-1} *Taq*) were added to each slide and coverslips were dropped. Slides were covered in mineral oil, and the following profile was run: 35 cycles of 2 min 55 $^\circ\text{C}$, 2 min 72 $^\circ\text{C}$, 2 min 94 $^\circ\text{C}$. Slides were placed in xylene to remove the mineral oil, and sections were re-hydrated. PCR product was detected *in situ* with an alkaline phosphatase-labelled anti-digoxigenin antibody, according to the manufacturer's instructions.

Unilateral substantia nigra lesioning. Unilateral nigral lesions were generated using the method of Perese *et al.*¹⁴ as described¹⁴. In brief, male Sprague Dawley rats, 290–310 g were anaesthetized with xylazine/ketamine and placed in a Kopf stereotaxic frame. The skull was exposed and burr holes drilled above the left substantia nigra, Lambda +3.5, L 2.15). Freshly made 6-OHDA (4 μg in 2 μl of 0.1% ascorbic acid in PBS) was loaded into a Hamilton syringe which was lowered into two sites over 2 min. The coordinates of the medial site were lateral 1.9 and ventral 7.1 mm with the needle bevel facing rostrally, whereas the lateral site was 2.3 mm lateral and 6.8 mm in the dorsal ventral plane with the needle bevel orientated laterally¹⁴. At each site 2 μl were injected over 5 min and the needle left in place for a further 5 min before being withdrawn over an additional 5 min.

Behavioural testing. Rats were tested 10–16 days following the 6-OHDA injections. They were placed in a hemispherical rotometer and the total number of complete body turns was recorded from 15–20 min following the administration of apomorphine (1 mg kg^{-1} intraperitoneally (i.p.)) as described¹⁵. A minimum of three tests separated by at least two weeks was used to generate a basal rotation rate. Animals which consistently exhibited stable (less than 25% variation) asymmetrical rotational behavior of greater than 10 turns per min were randomly selected for either AAVlac, AAVth or PBS injection.

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Stereotaxic injection of AAVlac or AAVth in 6-OHDA lesioned rats. Rats meeting the above behavioural criteria were anaesthetized with ketamine/xylazine (70 mg per 7 mg per kg i.p.) and placed in a Kopf stereotaxic frame. The skull was exposed and three holes drilled above the denervated striatum (left) at Paxinos & Watson¹² coordinates of anterior-posterior (AP) 0.2, lateral (L) 2.6 and AP 1.5, L 2.0 and L 3.0. Either AAVlac, AAVth or PBS was injected slowly using a Hamilton syringe into each of three sites at a DV depth of 5 mm. Each injection volume was 2 μl . Rats were tested for apomorphine-induced rotational behaviour at one and two months following surgery.

TH, NF and GFAP immunocytochemistry. For immunohistochemical (IHC) analysis of brain sections, rats were deeply anaesthetized with chloral hydrate and perfused with 1 M PBS (pH=7.3) followed by 4% paraformaldehyde (PF). Brains were removed and post-fixed (3–4 h) in 4% PF followed by ascending sucrose solutions (10/15/30% in PBS). Sections (7–30 μm) were cut in a cryostat (Reichert-Jung) and mounted on polylysine-coated slides. Sections were initially incubated in blocking buffer (5% Goat Serum (GS)/5% Normal Horse Serum (NHS) in 1 M Phosphate Buffer Saline (PBS)), followed by 2–4 h incubation at room temperature with mouse anti-TH primary antibody diluted in blocking buffer (Boehringer Mannheim, 1:200). After washing, anti-TH antibodies were detected with a Texas red-conjugated anti-mouse Ig antibody (Vector, 1:75). For double labelling, sections were again washed and then incubated with a second primary antibody, either mouse anti-NF (Sigma, 1:400) or rabbit anti-GFAP (gift from Dept. Pathology, Memorial Hospital, 1:800). Anti-NF, which was previously biotinylated, was detected by incubation at room temperature for 1 h with an avidin-FITC conjugate (Molecular Probes, 1:400). Anti-GFAP was detected by incubation with a biotinylated anti-rabbit IgG antibody (Vector, 1:400) following by detection with the avidin-FITC conjugate. Slides were coverslipped with PBS/Glycerol (0.05:1) and kept at -20°C .

Statistics. Between-group differences in apomorphine-induced rotation rates were evaluated using 3(group) \times 5(time) repeated-measures analyses of variance (ANOVA), which were calculated using the raw rotation rate data. Treatment associated changes (decrease) in the rotational behaviour were treated as statistically reliable when there was significant differences between AAVth-treated and the AAVlac and PBS-treated groups. The significant AAVth effects were further analysed using post-hoc tests including Fisher's Least Significant Difference (LSD) and Tukey tests, comparing values for each of the two time intervals following AAVth administration (4 weeks and 9 weeks) with both the PBS-treated and AAVlac-treated controls animals. All statistical results were generated using the Systat Ver. 5.2.1 software (Systat, Inc., Evanston, IL).

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